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RESEARCH ARTICLE

Native and modified lactate dehydrogenase expression in a fumaric acid producing isolate *Rhizopus oryzae* 99-880

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Abstract *Rhizopus oryzae* is subdivided into two groups based on genetic and phenotypic differences. Type-I isolates accumulate primarily lactic acid when grown in the presence of a fermentable carbon source and contain two lactate dehydrogenase genes, *ldhA* and *ldhB*. Type-II isolates synthesize predominantly fumaric acid and only have an *ldhB* gene. In this study, we determined that *ldhB* transcript is only minimally expressed in the Type-II isolate *R. oryzae* 99-880. LdhB enzyme purified from gene clones isolated from the Type-I isolate *R. oryzae* NRRL 395 and the Type-II isolate *R. oryzae* 99-880 each showed reductive LDH activity (pyruvate to lactate), while no oxidative LDH activity (lactate to pyruvate) was detected in either preparation. A transformation system was then developed for the first time with *R. oryzae* 99-880, using a uracil auxotrophic isolate that could be complemented with an orotate phosphoribosyltransferase gene, *pyrF*, isolated in this study. Transformation of this Type-II isolate with the *ldhA* gene from *R. oryzae* NRRL 395 resulted in reductive LDH activity between 1.0 and 1.8 U/mg total protein. Additionally, transformed isolates grown with glucose accumulated up to 27 g lactic acid/l with a concurrent decrease in fumaric acid, ethanol, and glycerol compared with the untransformed and vector-transformed control strains.

Keywords Rhizopus oryzae · Rhizopus arrhizus · Lactic · Fumaric · Lactate dehydrogenase · Orotate phosphoribosyltransferase · Transformation

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Introduction

Rhizopus oryzae (syn. *R. arrhizus*) is a ubiquitous organism in the family Mucoraceae that is frequently found in decaying organic matter rich in complex carbohydrates (i.e. plant material and animal excrement). This filamentous fungus has the ability to grow in such environments because of its simple growth requirements and capacity to produce numerous hydrolytic enzymes. Additionally, it is the most prevalent cause of zygomycosis, a life-threatening infection that usually afflicts immunocompromised patients or those with specific underlying abnormalities (Ribes et al. 2000; Ibrahim et al. 2003; Spellberg et al. 2005). However, because it is generally regarded as safe to those with a healthy immune system, it is commonly used for many industrial and food applications. In particular, considerable efforts have focused on the ability of this fungus to produce high levels of lactic acid or fumaric acid for chemical feedstock production from renewable resources.

Production of lactic acid (Saito 1911) and fumaric acid (Ehrlich 1911) by members of the Mucoraceae was first reported nearly a century ago. It was not until several decades later that *Rhizopus* was identified as one of the more proficient fungi for production of fumaric or lactic acid (Lockwood et al. 1936; Waksman and Hutchings 1937; Foster and Waksman 1939). Since then, numerous studies have focused on screening methods and fermentation optimizations to improve production of either of these products by *R. oryzae* (Magnuson and Lasure 2004). Metabolic studies have been useful in confirming that pyruvate can be directed into the TCA cycle or used fermentatively (Fig. 1) for the synthesis lactic acid, fumaric acid, or ethanol (Wright et al. 1996; Longacre et al. 1997; Bai et al. 2004). The synthesis of ethanol tends to occur primarily with anaerobic stress, presumably through the catalysis of pyruvate decarboxylase, Pdc, and alchohol dehydrogenase (Skory 2003), while aerobic growth favors production of organic acids (Margulies and Vishniac 1961). Conversion of pyruvate to lactic acid is known to proceed by way of an NAD-dependent L-lactate dehydrogenase (Obayashi et al. 1966; Pritchard 1973; Yu and Hang 1991). Work by Skory demonstrated that a *R. oryzae* strain capable of producing high levels of lactic acid had two NAD-dependent L-lactate dehydrogenase genes, *ldhA* and *ldhB* (Skory 2000). The enzyme LdhA was primarily responsible for conversion of pyruvate to lactic acid, when cells were grown in the presence of fermentative carbon sources, such as glucose or xylose. Conversely, transcript from the *ldhB* was only detected when the fungus was cultured on gluconeogenic substrates, such as glycerol, ethanol, or lactate. It was originally hypothesized that LdhB might have a role in the utilization of lactic acid, since oxidative NAD-dependent LDH activity was prevalent under these growth conditions. Fumaric acid is believed to be synthesized primarily in the cytosol by combined activities of pyruvate carboxylase, malate dehydrogenase, and fumarase (Osmani and Scrutton 1985; Kenealy et al. 1986; Peleg et al. 1989). Therefore, available pyruvate must be shared among competing pathways for lactic acid, ethanol, fumaric acid, or the TCA cycle. Efforts at strain improvement have successfully included mutatagenesis (Skory et al. 1998; Bai et al. 2004; Ge et al. 2004) and genetic modification (Skory 2004a) to enhance conversion efficiency of the specific routes towards a desired product.

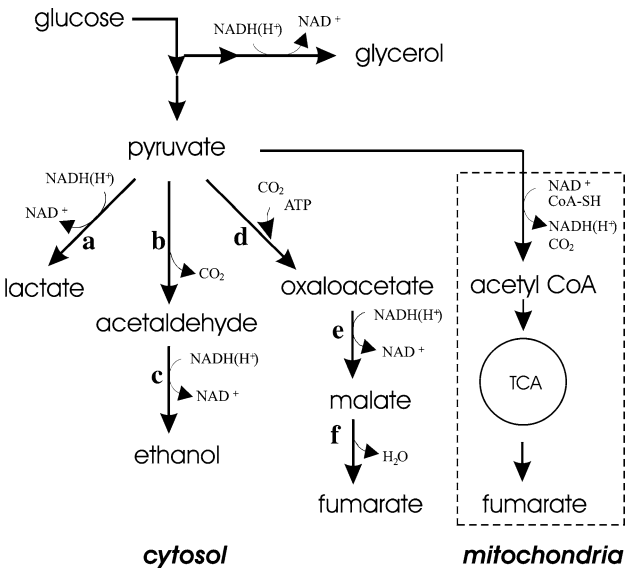

Fig. 1 Production of major fermentation products by *R. oryzae*. Enzymatic activities shown as: **a** lactate dehydrogenase; **b** pyruvate decarboxylase; **c** alcohol dehydrogenase; **d** pyruvate carboxylase; **e** malate dehydrogenase; **f** fumarase

It was recently discovered that significant taxonomic differences between *R. oryzae* strains partly explain which of these fermentative products are predominant. For example, Abe et al. demonstrated that *R. oryzae* strains could be separated into two groups, Types-I and II, based on rDNA ITS sequence (Abe et al. 2003). Type-I strains produced primarily lactic acid, while Type-II strains produce a predominance of fumaric acid. Differences between the type-strains were further differentiated when Saito et al. showed that Type-I isolates possess both *ldhA* and *ldhB* genes, but Type-II only have the *ldhB* gene (Saito et al. 2004). Many of the strains in the Type-II group do produce some lactic acid, suggesting that LdhB is likely responsible for reductive conversion of pyruvate to lactic acid in these isolates. The functionality of reductive NAD-dependent LDH activity for this enzyme is also supported by the ability of the *ldhB* gene from a Type-I *R. oryzae* isolate to restore lactic acid production in *Escherichia coli* mutants (Skory 2000). However, even with the ability to convert pyruvate to lactic acid, the predominance of fumaric acid in Type-II strains suggests an insufficient reductive capability of this enzyme. Excess pyruvate would then be available for conversion to fumaric acid or ethanol, thereby serving as an alternative route to regenerate NAD+.

The goal of this study was to first determine which conditions lead to transcription of *ldhB* in a *R. oryzae* Type-II isolate and to resolve questions about whether purified LdhB is even capable of converting pyruvate to lactic acid. We choose to focus these efforts on the Type-II isolate *R. oryzae* 99-880, because its genome has recently been sequenced as part of the Fungal Genome Initiative at the Broad Institute of Harvard and MIT. The LdhB protein from a Type-I isolate *R. oryzae* NRRL 395 is also included in this study for comparisons. Additionally, we were interested in determining whether genetic manipulation of *R. oryzae* 99-880 can shift organic acid synthesis to lactic acid production instead of fumaric acid by introducing an *ldhA* gene from a Type-I isolate. In this study, we also describe

the first transformation system in *R. oryzae* that utilizes complementation of the orotate phosphoribosyltransferase gene, *pyrF*, for introduction of genes of interest.

Materials and methods

Expression of *R. oryzae* LdhB protein in *E. coli*

The *ldhB* gene was cloned from DNA isolated from *R. oryzae* NRRL 395 and 99-880 (deposited in Fungal Genetic Stock Center as FGSC No. 9543) by PCR amplification with Pfu polymerase. Oligonucleotide primers, LdhB-P1 5′-ATG gta cta cat tca aag gtt gcc a-3' and LdhB-P2 5′-TTA tga taa ata ttc aac tgc ttt cag tg-3′, were designed to amplify only the coding region of the *ldhB* gene from each of the fungal isolates (start and stop codons capitalized in primer sequence). Amplified product was treated with Taq polymerase to add adenosine overhangs and cloned into either pCR2.1-TOPO (Invitrogen, Carlsbad, CA) for preparation of labeled hybridization probe or into pET-SUMO (Invitrogen) for expression in *E. coli*.

After confirmation of orientation and nucleotide sequence, LdhB protein was expressed in *E. coli* BL21 (DE3) according to manufacturer's recommendations. We lysed the bacterial suspension in PG Buffer (100 mM sodium phosphate, pH 6.8; 20% glycerol) using a French Press Cell pressurized to 17,000 psi. Clarified extracts of recombinant protein containing an N-terminal 6xHis tag and SUMO fusion for increased solubility were purified with Talon Cobalt Affinity Resin (Clontech, Mountain View, CA) according to suggested parameters. SUMO protease (Invitrogen, CA, USA) allowed cleavage of the N-terminal fusion adjacent to the putative LdhB protein. The cleaved 6xHis/SUMO fragment and uncut fusion protein could be removed with Talon Affinity Resin, so that only native LdhB with the naturally occurring N-terminal methionine passed through the column. The final LdhB proteins were stored in PG Buffer plus 5 mM dithiothreitol (DTT) to improve enzyme stability and stored at 4°C. Denaturing polyacrylamide gel (SDS-PAGE) electrophoresis was performed with 15% Tris-HCl Criterion Pre-Cast Gels (Biorad, Hercules, CA) according to manufacturer's suggestions. We performed protein detection with Sypro Red (Invitrogen) using recommended parameters for staining and photographing.

LDH activity was assayed spectrophotometrically by measuring the first-order change in absorbance at 340 nm as a result of oxidation of NADH or reduction of NAD+. Parameters for analysis are previously described (Skory 2000). Protein concentrations were determined by UV absorbance at 280 nm, using molar extinction coefficients of 23,170 and 23,295 for *R. oryzae* NRRL 395 and 99-880 LdhB, respectively. These values were calculated from the putative amino acid sequences of each protein according to the methods of Giles and von Hippel (Gill and von Hippel 1989). All assays were performed in triplicate and 1 unit of enzyme activity was defined as the amount of activity necessary to convert 1 μmol of NADH to NAD+ per minute or 1 μmol of NAD+ to NADH.

Development of fungal transformation system

A DNA fragment containing an orotate phosphoribosyltransferase gene (encoded by *pyrF*) was PCR amplified using Pfu polymerase with DNA isolated from *R. oryzae* 99-880. Primers PyrF-P1 5′-CGG ATT CGA AGC TTT AGG TCA A-3′ and PyrF-P2 5′-CTG CTG CTG CCA CTT ACT TTC TTA-3′ amplified a 2,066-bp fragment that was cloned into pCR2.1-Topo, following the addition of thymidine overhangs by Taq polymerase (Fig. 2). Inserts cloned into the plasmid in both orientations were isolated and fully sequenced. Plasmids with *pyrF* inserts in the opposite orientation to the *lacZ* cloning site were called pPyrF2.1-A, while those in the same orientation were designated pPyrF2.1-B.

R. oryzae 99-880 uracil auxotrophic mutants were obtained by first mutagenizing germinated spores with 1-methyl-3-nitro-1-nitrosoguanidine as previously described (Skory et al. 1998). Selection of isolates requiring uracil for growth on defined medium was then performed with 5-fluoroorotic acid (FOA), as before (Skory 2002). Only FOA-resistant isolates unable to grow on minimal medium lacking uracil, but capable of continued vigorous growth on medium with uracil were selected for study. We performed transformation of auxotrophic isolates with microprojectile particle bombardment (Biorad, CA, USA) using plasmid pPyr225 (Skory 2002) containing the *R. oryzae* NRRL 395 *pyrG* or plasmid pPyrF2.1-A (above). One of the auxotrophic isolates, *R. oryzae* M16, was chosen for reasons of stability and ease of complementation with plasmid pPyrF2.1-A. In order to determine the genotype of the mutation resulting in a defective orotate phosphoribosyltransferase, we PCR amplified the same 2,066 bp fragment containing the *pyrF* gene from *R. oryzae* M16 DNA using primers PyrF1/PyrF2 and compared the sequence back to the native gene.

Expression of *ldhA* in *R. oryzae* M16

Plasmid pLdhA-48XI which contains a *ldhA* gene fragment from *R. oryzae* NRRL 395 (Skory 2004a) was digested with *Cla*I and *Xma*I to remove the 2.28-kb *pyrG* gene fragment used for selection (Fig. 2). The remaining vector containing a 3.3-kb *ldhA* fragment was treated with T4 polymerase to remove restriction endonuclease overhangs. We then ligated the 2.0-kb *pyrF* fragment obtained from an

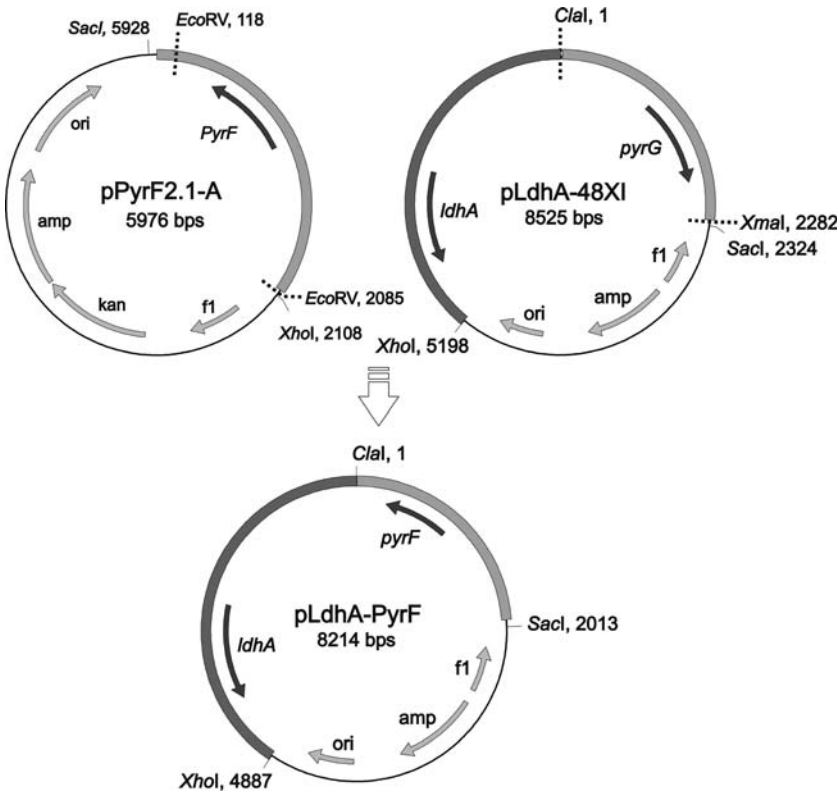

Fig. 2 Design of plasmid pLdhA-PyrF. A DNA fragment containing the *pyrG* gene was removed from plasmid pLdhA-48XI by enzymatic digestion with *Cla*I and *Xma*I. Following T4 polymerase treatment to remove overhangs, the 1.97-kb *pyrF* fragment released from plasmid pPyrF2.1-A by *Eco*RV digestion was subsequently ligated in its place. *Shaded thick lines*, *R. oryzae* DNA; *shaded arrows*, coding regions

*Eco*RV digestion of the pPyrF2.1-A plasmid to obtain the resultant plasmid pLdhA-PyrF. This plasmid was then transformed into *R. oryzae* M16 using biolistic methods previously described. Vector plasmid pPyrF2.1-A was also transformed into *R. oryzae* M16 and served as one of the controls. Approximately 5–7 days following bombardment, spores were collected and diluted in sterile water to obtain single-spore isolates. Only one isolate per plate was selected to avoid multiple progeny originating from the same transformation.

Four different *R. oryzae* M16 isolates transformed with pLdhA-PyrF were used for further analysis. *R. oryzae* 99-880 and *R. oryzae* M16 transformed with PyrF2.1-A served as controls. In order to determine the rate of sugar utilization and accumulation of fermentation products, we grew cultures in shake-flasks, in triplicate, as described previously (Skory 2004a). The fermentable carbon source in the RZ culture medium consisted of 100 g glucose/l, with 30 g calcium carbonate/l added for pH control. The dry weight of the mycelium was calculated at the end of the fermentation by solubilizing excess calcium carbonate in 0.75 M HCl prior to filtering and drying the fungal biomass. Lactic acid, fumaric acid, glycerol, ethanol, and glucose concentrations in the medium were analyzed by HPLC using refractive index detection as previously described (Skory 2000).

Cultures for enzymatic analyses were grown in a similar manner, except only 5 g glucose/l was included into the medium to minimize acid production and cultures were grown for a period of 24 h with orbital shaking at 200 rpm at 30°C. Mycelium was collected by filtration and disrupted with glass beads in a similar manner to that described previously (Skory 2000). Lysates cleared by centrifugation were immediately tested for enzymatic activities. Conditions for pyruvate decarboxylase (PDC) assays are described elsewhere (Skory 2003). Malate dehydrogenase (MDH) assays were measured in a similar manner using 50 mM sodium phosphate buffer (pH 7.4) and 0.25 mM NADH equilibrated to 30°C. Reactions were initiated by the addition of oxaloacetate to a final concentration of 0.2 mM. Protein concentration of lysates was determined using Bio-Rad protein reagent, with bovine serum albumin as standard.

Probability analysis for fermentation and enzymatic studies was performed using the Student's *t*-test with 2-tailed distribution. Data for all sample sets were compared with the *R. oryzae* 99-880 control strain. Values with $p < 0.05$ were considered significant for this study and are included with presentation of data.

Molecular analyses

Northern hybridization analysis of *R. oryzae* 99-880 grown in the presence of different carbon sources was used to detect induction conditions of *ldhB* expression. *R. oryzae* 99-880 was inoculated at a concentration of 2×10^3 spores/ml of RZ medium supplemented with 0.1 g trypticase

peptone (BBL, Sparks, MD)/l and 5 g/l of the following carbon sources: glucose, potato starch, xylose, mannose, trehalose, glycerol, and ethanol. Flasks were incubated 18 h at 30°C with orbital shaking at 250 rpm. We isolated RNA from harvested mycelium using a hot phenol method (Schmitt et al. 1990), followed by further purification with RNeasy cleanup (Qiagen, Valencia, CA). Confirmation of RNA quality was determined using an Agilent 2100 Bioanalyzer (Santa Clara, CA). Only RNA samples having an integrity number (RIN) >8 were considered sufficient for further analysis. Full length digoxigenin-labeled (Roche, Indianapolis, IN) RNA transcript was prepared from *ldhB* gene cloned into PCR2.1-Topo (above) in the anti-sense orientation to T7 promoter and used as probe for northern analysis. Quality of RNA probes was confirmed using the Bioanalzer prior to using for hybridizations. Detection of *ldhB* RNA was performed using RT-PCR amplification with primers specific to *ldhB* sequence in a manner similar to that previously described (Skory 2000). Amplification was also performed with RNA that was not treated with reverse transcriptase to serve as a control to determine if amplification product was from DNA contamination.

Southern analysis of *R. oryzae* 99-880 and subsequent transformant isolates was performed similar to conditions previously described, except fungal DNA was digested with *Hpa*I (Skory 2005). The *pyrF* probe used for hybridization was an internal 582-bp PCR fragment obtained with PyrF-P3 5′-TGC ACT TGC CAA TGA TGT CTT A-3′ PyrF-P4 5′-CAA AGC CAA TTC AGC CTC AAA TG-3′ and labeled with digoxigenin dUTP. The ldhA probe used for hybridization was an internal 1,000 bp PCR-labeled *ldhA* fragment described previously (Skory 2004a). All detections of hybridization blots were performed with an Image Station 1000 (Kodak, Rochester, NY).

Results

Expression of *ldhB* in *R. oryzae* 99-880 and *E. coli*

Northern analysis was performed on RNA isolated from cultures grown on several different carbon sources, because it was not known whether *ldhB* was expressed in *R. oryzae* 99-880. However, no signal could be detected even when using labeled RNA probes. In contrast, PCR amplification primers specific to *ldhB* sequence and cDNA synthesized from each of the RNA samples did result in formation of *ldhB* product for all of the samples (data not shown). We confirmed that this expression was not due to DNA contamination because signals could not be detected when samples not treated with reverse transcriptase were used in the PCR.

The *ldhB* genes from *R. oryzae* NRRL 395 and 99-880 were then cloned into an *E. coli* expression vector in order to determine if they encoded functional enzymes. The coding region of *ldhB* from both strains could be accurately amplified with the same set of primers, LdhB-P1/P2, because of the high degree of sequence identity, 96.7%, between these genes. The addition of the 6x His Tag to the recombinant protein facilitated purification from the *E .coli* lysate, while the SUMO protease allowed proteolytic cleavage of the amino terminal fusion. Denaturing gel electrophoresis of the final purified LdhB proteins confirmed an approximate size of 32 kDa (Fig. 3), suggesting that SUMO protease had cleaved the translation fusion protein at the probable recognition site, resulting in a recombinant protein with identical sequence to that of the putative native LdhB. Enzymatic analysis of this protein revealed a specific reductive Ldh activity of 21.4 (±0.7) and 9.1 (±0.8) units/mg protein for LdhB from *R. oryzae* NRRL 395 and 99-880, respectively. We were not able to detect any oxidative Ldh activity for either protein.

Development of a transformation system

Approximately 20 different isolates derived from *R. oryzae* 99-880 were identified after mutagenesis and selection on medium containing FOA. However, most of these were unable to maintain continued growth on medium with the selectable agent. Furthermore, only one of the isolates, *R. oryzae* M16, exhibited a stable phenotype for uracil auxotrophy. No revertants appeared on medium lacking uracil, even when plating more than 1×10^9 spores from *R. oryzae*

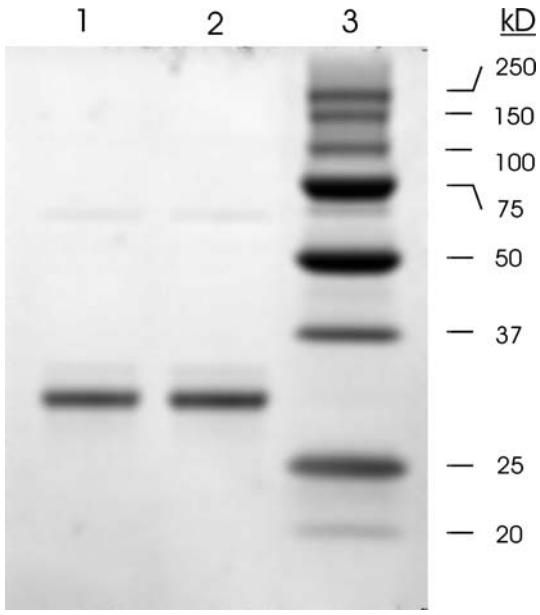

Fig. 3 SDS-PAGE (15%) of purified LdhB protein. Approximately 10 μg each of LdhB from *R. oryzae* NRRL 395 and 99-880 were separated in *Lanes 1 and 2*, respectively. *Lane 3* contains Precision Plus Protein Standards (Biorad) with MW shown in kD

M16. Additionally, *R. oryzae* M16 did not grow on minimal medium supplemented with orotidine, and transformation of this strain with plasmid pPyr225 containing the OMP-decarboxylase gene, *pyrG*, from *R. oryzae* NRRL 395 was unable to restore prototrophy. Therefore, it was hypothesized that the auxotrophic mutation was likely in the orotate phosphoribosyltransferase gene, *pyrF*, so efforts were directed at isolation of this gene.

Using published genome sequence, we designed primers to PCR amplify a 2,066-bp fragment containing the *pyrF* gene, which was subsequently cloned into the pUC-derived plasmid pCR2.1-TOPO. Sequence analysis of the resultant plasmids having each orientation of the insert showed that the cloned *R. oryzae* DNA fragment was identical to that available through the Broad Institute of Harvard and MIT, except for a discrepancy of two deoxynucleotides. A string of thymidines located 40-bp upstream of the *pyrF* gene was described by Broad as being 24 bp in length, while plasmids pPyrF2.1-A and pPyrF2.1-B, had 23 and 21 thymidines, respectively.

Sequence comparison with other orotate phosphoribosyltransferases suggests that the *pyrF* gene is encoded on the 2,066 bp fragment as a single open reading frame between 998 and 1651. The putative 218 amino acid protein is most similar to the orotate phosphoribosyltransferase from *Mucor circinelloides* with a similarity index in excess of 85% by Lipman–Pearson alignment methods (Altschul et al. 1990). *M. circinelloides* also had a comparable upstream thymidine region at −151 that was 26-bp long and also included several cytidines. The greatest region of nucleotide sequence identity with the *M. circinelloides pyrF* gene was in excess of 73% and spanned the region from the thymidine repeat to the stop codons of the orotate phosphoribosyltransferase genes.

The majority of the upstream region of the *R. oryzae pyrF* gene fragment appears to encode for part of a zinc binding alcohol dehydrogenase superfamily (Babiychuk et al. 1995). Sequence alignments of this upstream region with other proteins in this family indicate that the coding region for this gene ends just 14 bp upstream of the thymidines stretch. This results in a span of only 76 bp between the end of the putative oxidoreductase and the start of the orotate phosphoribosyltransferase. Interestingly, there also appears to be a similar oxidoreductase coding region upstream of the *M. circinelloides pyrF* gene that has 60% sequence identity with the equivalent region in *R. oryzae*.

In order to determine why *R. oryzae* M16 was unable to grow on medium lacking uracil, we PCR amplified the same 2,066 bp region containing the *pyrF* gene and compared the sequence with *R. oryzae* 99-880. The only difference in the entire fragment was a single G to A nucleotide transversion at nt +205. This mutation would result in changing amino acid glycine 69 to an aspartic acid.

Transformation of plasmid pPyrF2.1-A into *R. oryzae* M16 by biolistic methods fully restored prototrophic growth on minimal medium. Southern analysis of multiple isolates confirmed that the *pyrF* containing plasmid was replicating autonomously in transformants (data not shown). Transformation efficiency was not tested, but appeared to be comparable to that previously described for auxotrophic isolates derived from *R. oryzae* NRRL 395.

Fermentations with modified *R. oryzae* isolates

We were also interested in determining whether *ldhA* from *R. oryzae* NRRL 395 could be expressed in *R. oryzae* M16, so we replaced the *pyrG* from a previously described *ldhA* containing plasmid, pLdhA-48XI, with the *pyrF* gene (Fig. 2). Plasmid pLdhA-48XI contains a 3.3-kb *Bsm*I genomic fragment from *R. oryzae* NRRL 395 that is capable of expressing LdhA from the native promoter. The resultant plasmid, pPyrF-LdhA, was then transformed into *R. oryzae* M16 using biolistic methods. Four different transformed isolates, designated LdhA-PyrF Nos. 1–4, were chosen for further study. Additionally, a single isolate obtained by transformation with plasmid pPyrF2.1 without *ldhA* and the parent strain *R. oryzae* 99-880 were included as controls.

These strains were tested with shake flask fermentation studies to determine the rate of sugar utilization and the accumulation of fermentation products. Compared with fermentation performed in a similar manner with *R. oryzae* NRRL 395 (Skory 2004a), transformed isolates and controls were approximately three times slower at utilizing the available carbon source. The rate of glucose utilization was slightly higher for transformants LdhA-PyrF Nos. 1 and 2, compared with the remaining isolates. This difference was most noticeable at 72 h after inoculation, when LdhA-PyrF Nos. 1 and 2 had utilized 12 and 32%, respectively, more glucose than the untransformed control (data not shown). However, it still required ~6 days of growth until all of the isolates had utilized at least 95% of the sugar.

The general trends for accumulation of fermentation products remained consistent throughout the fermentation. Therefore, only the final concentrations of secreted metabolites from the end of the fermentation are presented (Table 1). Three of the isolates transformed with plasmid pLdhA-PyrF accumulated lactic acid throughout the fermentation. Isolates LdhA-PyrF Nos. 1 and 2 had the highest yield of lactic acid, with a total accumulation of 16.2 and 26.9 g/l, respectively. We were unable to detect any lactic acid (limit of detection ~0.1 g/l) for untransformed 99-880 control or the PyrF transformed control throughout the fermentation. Additionally, one of the LdhA-PyrF isolates, No. 4, did not have any detectable lactic acid throughout the fermentation.

All of the isolates producing lactic acid accumulated less fumaric acid, ethanol, and glycerol than the *R. oryzae*

Table 1 Accumulation of fermentation products and biomass by *R. oryzae* isolates

Strain	Accumulated product (g/l)[a]				Mycelial dry Weight (g/flask)[b]
	Lactic acid	Fumaric	Ethanol	Glycerol	
LdhA-PyrF No. 1	16.2 (0.1) $p = 0.000$	18.6 (1.0) $p = 0.050$	20.2 (1.6)	2.9 (0.0) $p = 0.044$	0.75 (0.11) $p = 0.010$
LdhA-PyrF No. 2	26.9 (0.6) $p = 0.000$	16.8 (0.5) $p = 0.034$	15.8 (0.9) $p = 0.018$	2.7 (0.1) $p = 0.029$	0.75 (0.03) $p = 0.003$
LdhA-PyrF No. 3	8.5 (0.1) $p = 0.000$	20.6 (1.3)	24.0 (1.3)	2.9 (0.1)	0.92 (0.07) $p = 0.006$
LdhA-PyrF No. 4	0	21.0 (1.3)	27.8 (1.6)	3.2 (0.4)	0.96 (0.12)
PyrF control	0	25.6 (4.5)	31.8 (2.2) $p = 0.000$	2.0 (0.3) $p = 0.006$	1.23 (0.05) $p = 0.015$
99-880 Control	0	22.7 (1.2)	24.5 (1.8)	3.3 (0.1)	1.02 (0.05)

Values are from samples obtained 144 h after inoculation, when glucose was >95% utilized

[a] Standard deviation between triplicates shown in parentheses. The *p*-value is also included for samples differing from 99-880 control strain with probability factor ≤ 0.05

[b] Flasks contained 50 ml growth medium

99-880 control strain. Additionally, these isolates also had less mycelial dry weight at the end of the fermentation. This proportional decrease in fermentation byproducts with lactic acid production was most evident with isolate LdhA-PyrF No. 2, which had the highest amount of lactic acid. This transformant accumulated 26% less fumaric acid, 36% less ethanol, and 18% less glycerol when compared with the *R. oryzae* 99-880 control. There was no statistical difference ($p \geq 0.05$) in accumulated levels of any of the fermentation products or mycelial dry weight for LdhA-PyrF No. 4 compared with *R. oryzae* 99-880 control. Interestingly, the PyrF transformed control strain accumulated 30% more ethanol, 39% less glycerol, and had 20% more mycelial dry weight than the *R. oryzae* 99-880 control strain. Levels of malic acid generally paralled accumulated fumaric acid for all of the isolates, except that concentration was typically less than 1 g/l and accurate measurements were difficult to obtain (data not shown).

Enzymatic analysis of modified *R. oryzae* isolates

We tested protein extracts from the modified *R. oryzae* isolate for several enzymatic activities. Reductive LDH activity was detected only in protein extracts from transformants LdhA-PyrF Nos. 1-3 (Table 2). Even though LdhA-PyrF No. 3 had the highest average LDH activity of the three isolates, the variability was high enough that it was difficult to make conclusions about difference in activity between these three isolates. No reductive LDH activity was detected for LdhA-PyrF No. 4 and the two control isolates, PyrF and 99-880. Oxidative LDH activity was highest for LdhA-PyrF Nos. 1–3 and may be from expressed LdhA protein. The remaining three isolates, LdhA-PyrF No. 4 and the two control strains, had almost the same level of oxidative LDH activity.

All of the LdhA-PyrF isolates produced approximately 21% less PDC activity than the *R. oryzae* 99-880 control, but only isolates Nos. 1 and 3 were statistically significant. The PyrF control strain produced the highest amount of ethanol and had almost 50% more PDC activity than the average of the other isolates. Conversely, this isolate also had the lowest level of MDH activity. The remaining isolates did show some differences in average MDH activity. However, it was difficult to make any conclusions because of the variability among all of the isolates.

Southern analysis of modified *R. oryzae* isolates

Southern analysis of *R. oryzae* isolates using Dig-labeled *pyrF* as probe showed that all but one of the transformants

Table 2 Enzymatic activities (units/mg protein) of mycelial extracts of *R. oryzae* isolates

Strain	Reductive LDH	Oxidative LDH	PDC	MDH
LdhA-PyrF No. 1	1.54 (0.11) $p = 0.001$	0.45 (0.01) $p = 0.007$	0.62 (0.03) $p = 0.029$	10.8 (0.6) $p = 0.002$
LdhA-PyrF No. 2	1.02 (0.19) $p = 0.010$	0.39 (0.04) $p = 0.026$	0.64 (0.09)	11.6 (1.2) $p = 0.008$
LdhA-PyrF No. 3	1.84 (0.56) $p = 0.029$	0.67 (0.04) $p = 0.007$	0.67 (0.09) $p = 0.048$	11.8 (0.9) $p = 0.044$
LdhA-PyrF No. 4	0.04 (0.02)	0.23 (0.04)	0.69 (0.11)	14.1 (0.9) $p = 0.023$
PyrF control	0.01 (0.01)	0.22 (0.02) $p = 0.005$	1.03 (0.04) $p = 0.003$	8.4 (0.9) $p = 0.004$
99-880 Control	0.00 (0.02)	0.28 (0.01)	0.82 (0.04)	15.8 (0.9)

Standard deviation between triplicates shown in parentheses. The *p*-value is also included for samples differing from 99-880 control strain with probability factor ≤ 0.05

had plasmid replicating autonomously in high molecular weight concatenated structures (Fig. 4). This type of replication is typical with *Rhizopus* and usually results in a Southern hybridization with the majority of plasmid being detected above 23.1 kb or retained in the well, if the plasmid is not linearized. In this particular case, the restriction enzyme *Hpa*I which does not cut the plasmid was used to digest DNA prior to electrophoretic separation. The 5.3 kb band present in all isolates correlates with the anticipated fragment that would result from *Hpa*I digestion of the unmodified *pyrF*, suggesting that no integration occurred at the *pyrF* locus in these transformants. Such integration would result in a shift in size for the 5.3 kb *pyrF* fragment. Transformant isolate LdhA-PyrF No. 4 had no evidence of plasmid replication or integration and is likely the result of a gene conversion (Skory 2004b). Southern hybridization with *ldhA* probe resulted in a similar hybridization pattern as before for LdhA-PyrF isolates Nos. 1–3. The probe also hybridized to the genomic *ldhB*, because there is >90% identity between the *ldhA* and *ldhB* genes. However, the native *ldhB* fragment with *Hpa*I digestion was >23.1 kb and co-migrated with the signals from the plasmid. Therefore, we could only detect hybridization from *ldhB* in isolates lacking plasmid containing the *ldhA* gene; LdhA-PyrF No. 4, PyrF control, and 99-880 control (data not shown).

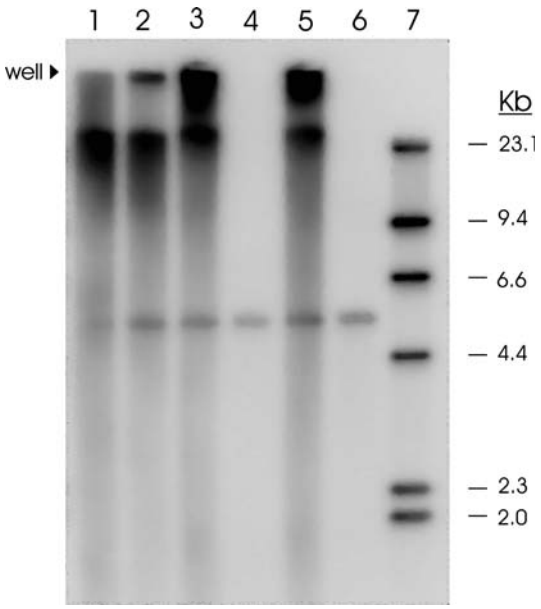

Fig. 4 Southern hybridization analysis *Hpa*I-digested DNA from recombinant *R. oryzae* isolates and control strains. An internal region of the *pyrF* coding region was used as probe. Labeled *Hind*III fragments of Lambda DNA were used as molecular weight standards and their sizes, in kilobases, are shown on the right. *The arrow* on the left marks the location of the wells. *Lanes 1–4*, Transformants LdhA-PyrF isolates Nos. 1–4; *Lane 5*, Transformant PyrF-control; *Lane 6*, *R. oryzae* 99-880 control; *Lane 7*, Standard

Discussion

Saito et al. noted that there were significant differences between the *R. oryzae ldhB* genes from each Type group. By comparing 48 different *R. oryzae* strains, they found that the *ldhB* gene in the Type-I isolates mutated at a higher rate than either the *ldhA* gene or the *ldhB* gene from the Type-II group (Saito et al. 2004). We further noticed that these mutations were scattered throughout the *ldhB* gene and resulted in amino acid modifications at 35 different positions for Type-I isolates. This contrast with the *ldhB* gene from the Type-II group which had greater than 99% identity among the 21 isolates and amino acid modifications were localized to only six different positions. We could not distinguish any pattern in amino acid substitutions for the 9 out of 21 Type-II strains that Saito et al. found were able to produce 0.2–2.4 g lactic acid/l. However, two amino acids positions clearly distinguished the LdhB proteins between each group. All LdhB from Type-I isolates had Ile37 and Arg153, while Type-II isolates had Val37 and Cys153. In fact, these two amino acids were the only differences between the LdhB from *R. oryzae* NRRL 395 and *R. oryzae* 99-880 used in this study.

We were particularly interested in the residue Arg/Cys153 difference which corresponds to Arg173 in other LDH, when numbered according to Eventoff et al. (Eventoff et al. 1977). The amino acid Arg173 is highly conserved among all LDH proteins and the presence of a cystein in this conserved position is exceptionally rare. It has been shown that Arg173 is directly involved in the binding of fructose 1,6-bisphosphate (FBP) in *Bacillus stearothermophilus* (Clarke et al. 1987; Cameron et al. 1994) and *Thermus caldophilus* (Matsuzawa et al. 1988). The glycolytic intermediate FBP accumulates in the presence of excess glucose and serves as an allosteric regulator to stimulate LDH function with numerous prokaryotes (Garvie 1980). This type of regulation is typically associated only with bacterial LDH and there is only one known example of a eukaryotic LDH showing allosteric effects with FBP. Purified LDH protein from the closely related Mucorales fungus *Phycomyces blakesleeanus* was found to have a higher tolerance for pyruvate inhibition at pH 6.0 when FBP was included in reactions (de Arriaga et al. 1982). It is unknown whether activity from either *R. oryzae* LdhB protein used in this study is modified by the presence of FBP.

Enzymatic analysis of the LdhB proteins from each *R. oryzae* group confirms for the first time that they are capable of converting pyruvate to lactate using NADH as cofactor. Additionally, the inability to detect LDH oxidative activity using NAD+ is indicative of preferential reductive catalysis toward the formation of lactate. This obviously raises doubts about whether LdhB is involved in the oxidative conversion of lactic acid during growth on gluconeo-

genic substrates and further suggests that it likely functions in vivo for the production of lactic acid. However, it is not entirely clear whether LdhB enzyme is even expressed in *R. oryzae* 99-880. We have not detected lactic acid in this particular Type-II isolate. Furthermore, we could only detect *ldhB* transcript by RT-PCR using RNA isolated from several different growth conditions, so any level of expression would be expectedly low. In previous work, we were unable to detect *ldhB* by northern analysis in the lactic acid producing *R.oryzae* NRRL 395 and could only detect transcript by RT-PCR. We believe that transcriptional regulation of the *ldhB* is the most likely reason why lactic acid was not detected in *R. oryzae* 99-880. It is also probable that some Type-II isolates are able to produce small amounts of lactic acid (Saito et al. 2004) because of increased transcription of the *ldhB* gene.

It then seemed logical to question whether the introduction of the *ldhA* gene into a *R. oryzae* Type-II isolate would allow production of lactic acid comparable to that of a Type-I isolate or if additional regulation of other genes (e.g. lactic acid transporters) were necessary. We first had to develop a transformation system in a Type-II isolate in order to test this idea. Auxotrophic selection based on uracil biosynthesis provides the most well characterized system available for *R. oryzae* (Skory 2002, 2004b, 2005; Michielse et al. 2004) and was chosen for use with *R. oryzae* 99-880. In previous work, *R. oryzae* mutants isolated in the presence of 5-fluoroorotic acid typically were an even mix of those defective in orotate phosphoribosyltransferase (encoded by *pyrF*) or OMP-decarboxylase (encoded by *pyrG*). In this particular study, only one stable uracil auxotroph was isolated and it was incapable of utilizing orotidine or being complemented by a previously isolated *pyrG* gene. We therefore used sequence available through the Broad Institute of Harvard and MIT to obtain the *pyrF* gene for use as a selectable marker. The *pyrF* fragments used for *R. oryzae* complementation in this study and by Velayos et al. (1998) for *M. circinelloides* transformation were both greater than 2 kb and included similar length of upstream region. While it is possible that some regulatory regions are encoded upstream of the thymidine–cytidine stretch, the transcription start site obtained from cDNA sequence for the *M. circinelloides* and *R. oryzae pyrF* are only −12 and −4, respectively. Therefore, it is likely that DNA upstream of the thymidines stretch can be removed for complementation studies, resulting in a considerably smaller size for the selectable marker.

The *R. oryzae* M16 uracil auxotrophic mutant isolated in this study was found to have a G/A nucleotide transversion in the *pyrF* gene that should result in changing amino acids Gly69Asp. Even though this was only a point mutation, we have never detected reversion occurring in any of our work. The amino acid substitution in this mutant is located in a region of the protein that is implicated in pyrophosphate binding in similarly related orotate phosphoribosyltransferase enzymes. Structural data from *Escherichia coli* (Henriksen et al. 1996) and *Salmonella typhimurium* (Scapin et al. 1995) reveals that there is a short flexible loop 69-GPAYKG-74 that allows Lys73 to interact with the beta-phosphate oxygen in the pyrophosphate (Ozturk et al. 1995). This amino acid sequence is highly conserved among almost all orotate phosphoribosyltransferase proteins and it is likely that the flanking glycines provide flexibility for this short loop. There are no published data on the effects of mutating Gly69. However, modification of the second glycine in a similar pyrophosphate binding guanine phosphoribosyltransferase protein completely inactivated the enzyme (Page et al. 1999).

Transformation of *R. oryzae* M16 with plasmid containing the *pyrF* gene resulted in restoration of prototrophic growth. This is the first description of a transformation system in *R. oryzae* based on complementation of the orotate phosphoribosyltransferase gene. As is typical with *R. oryzae* transformation, almost all of the isolates showed evidence of plasmid replicating autonomously in multi-copy high molecular concatenated structures (Skory 2002, 2005). Unlike most filamentous fungi, integration of DNA used for transformation of *Rhizopus* occurs only rarely. Even then, integration events are typically associated with gene conversion or replacement of the selectable marker (Michielse et al. 2004; Skory 2004b) as is seen with LdhA-PyrF isolates No. 4.

Transformation and replication of plasmid pLdhA-PyrF in *R. oryzae*, as is seen with isolates Nos. 1–3, conferred the ability to convert more than 25% of the starting glucose into lactic acid. Concomitantly, this shift in pyruvate conversion was also associated with a decreased accumulation of the fermentation products fumaric acid and ethanol. Furthermore, these isolates also showed a decreased mycelial dry weight with this pyruvate shift towards lactic acid. These results clearly demonstrate that the *ldhA* gene is functionally expressed in the Type-II isolate *R. oryzae* M16 without the need for any additional regulatory elements. Furthermore, this isolate is capable of exporting lactic acid into the medium through either facilitated diffusion or some other transport mechanism. It is possible that accumulation of lactic acid could be a result of expression from *ldhB*, since some Type-II isolates are capable of producing lactic acid. However, this possibility seems unlikely when neither reductive LDH activity nor lactic acid accumulation has ever been detected in *R. oryzae* 99-880 and more than 10-fold lactic acid was produced by the transformant isolates in this study when compared to the other Type-II isolates described by Saito et al. (2004). Differences in glycerol accumulation with the LdhA-PyrF isolates Nos. 1–3 and the PyrF control compared with the *R. oryzae* 99-880

control were more difficult to interpret. It is known from studies with yeast that glycerol formation is necessary for utilizing excess NADH during anaerobic conditions, since ethanol production is a redox neutral process (Nordstöm 1968; Van Dijken and Scheffers 1986). Even though *R. oryzae* fermentations are performed with aeration, oxygen limitation due to inadequate air transfer and mycelial clumping are unavoidable. Providing an additional route to regenerate NAD+ through expression of the LDH could result in less available co-factor for the production of glycerol during anaerobic metabolism (Nissen et al. 2000). However, this does not explain the decreased glycerol accumulation that occurred for the PyrF control.

The specific reductive LDH activities for the *R. oryzae* pLdhA-PyrF isolates Nos. 1–3 (Table 2) were comparable to typical activities observed with the more efficient lactic acid producing isolate *R. oryzae* NRRL 395 (Skory 2004a). We believe that the elevated level of oxidative LDH activity for these same isolates is likely from the expressed LdhA protein and not representative of increased expression of another enzyme. We do not know whether there is any significance to the presence of background oxidative LDH activity for the remaining isolates. The data in this study suggests that LdhB has very little oxidative activity in vitro and there are not any additional NAD+ linked LDH proteins that we can identify in the genome database. We are hesitant to make speculations based on comparisons between purified enzymes and crude cell homogenates. There did not seem to be any correlation between heterologous expression of LdhA and the level of PDC or MDH activity. This suggests that decreased accumulation of ethanol and fumaric acid in these isolates was a result of competition for pyruvate and not because of decreased expression of PDC or MDH.

We believe that this work further helps to explain differences in accumulation of fermentation products by *R. oryzae*. It has finally been established that the *ldhB* gene encodes for functional enzyme for the two isolates used in this study and that catalytic activity of LdhB is heavily balanced towards the formation of lactic acid. Additionally, we have clearly demonstrated that isolates derived from this *R. oryzae* 99-880 are capable of accumulating significant amounts of lactic acid with expression of the *ldhA* gene. Therefore, the inability to synthesize lactic acid in this Type-II isolate is likely related to levels of *ldhB* transcription/translation or possibly unfavorable kinetic properties of the enzyme for competing with available pyruvate. The results of this study suggest that the lack of lactic acid for *R. oryzae* 99-880 is likely due to negligible transcription of the *ldhB* gene.

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